Order of activities	Help Assay	TaqMan Assay
Activity 1	PCR amplification of ligated DNA	
Activity 2	Run *Hpa*II and *Msp*I-digested samples from Practical Class 1	
Activity 3		Receive TaqMan results, class discussion (please bring laptops)

What is the concentration of DNA in this reaction?

Procedure:

Step 1: The double-stranded adaptor will be provided at a concentration of 5μM

Step 2: each ligation will be done in a final total volume of 20μl with 200ng of *Hpa*II or *Msp*I-digested DNA

Step 3: Prior to adding the T4 DNA ligase, the double-stranded sequencing adaptor must be hybridized to the digested DNA. This is done by using a PCR thermocycler to heat the sample to 55°C and then cool it to 22°C over 1 hour

Step 4: Add 1μl of T4 DNA ligase and incubate for 60 minutes at 22°C

Table 5 Ligation of Sequencing adaptor to digested genomic DNA

Components	**Amount**	**Volume (to 20μl)**
*Msp*I or *Hpa*II-digested gDNA	200ng	
Sequencing Adaptor (5μM)	You will add adaptor to a final concentration of either 0.5 or 1μM – please ask the laboratory supervisor	
5X Rapid ligation buffer	1X in final reaction	
Water to a final volume of 19μl	-	
Before adding the T4 DNA ligase, heat the sample to 55°C and cool to 22°C over 1 hour		
T4 DNA ligase 5units/μl	5 units in the final reaction	

How many microlitres of each reaction component do we need?

Procedure:

Step 1: Using the primer AdaptorSEQ_FWD you will amplify the ligated gDNA
Step 2: Based on each ligation reaction containing 200ng of template DNA, the PCR amplification will include 20ng of ligated-*Msp*I-digested and 40ng of ligated-*Hpa*II-digested template

Table 6. PCR amplification of ligated gDNA

Components for PCR	**Stock concentrations**	**Amount or final concentration in reaction - *Hpa*II**	**Amount or final concentration in reaction - *Msp*I**	**µl in a reaction volume of 25µl**	
				***Msp*I**	***Hpa*II**
Digested and Ligated Template DNA					
MyTaq	2X	1X	1X		
AdaptorSEQ_FWD	5µM	0.5µM	0.5µM		
Nuclease-free water	-	-	-		

Which sample has the highest starting amount of template?

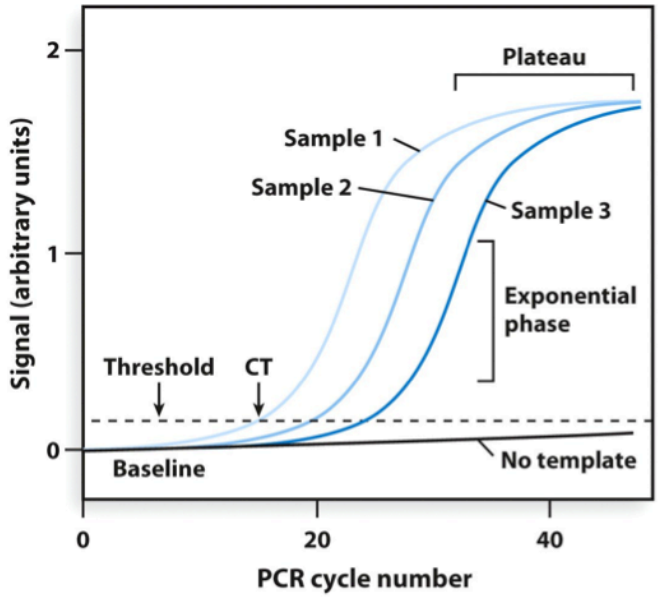

Figure 7-10
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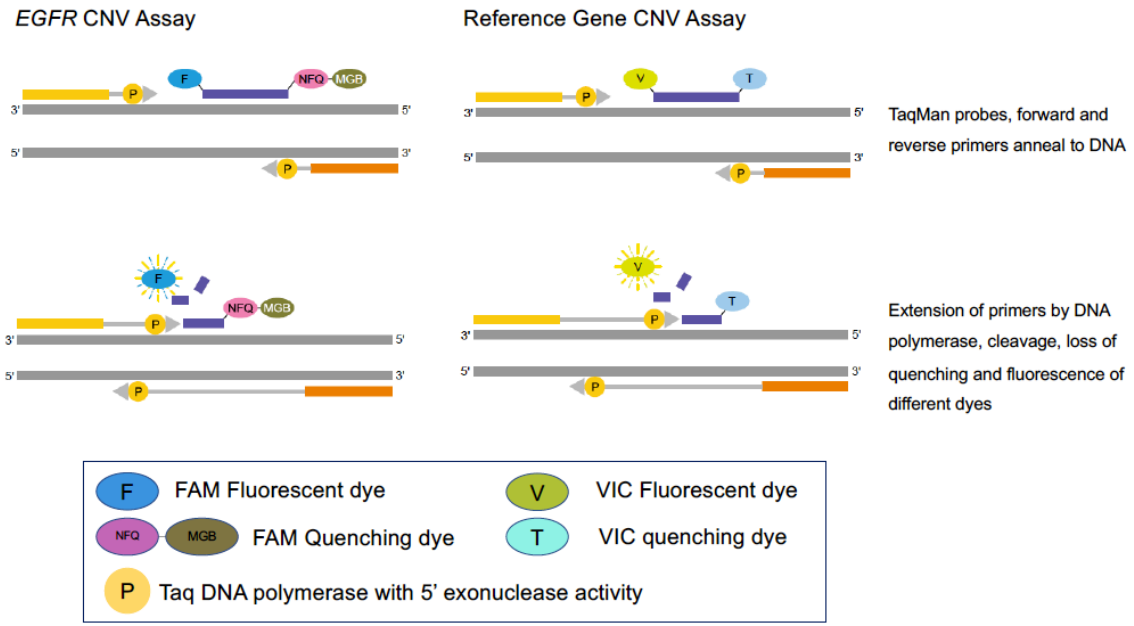
TaqMan Assay

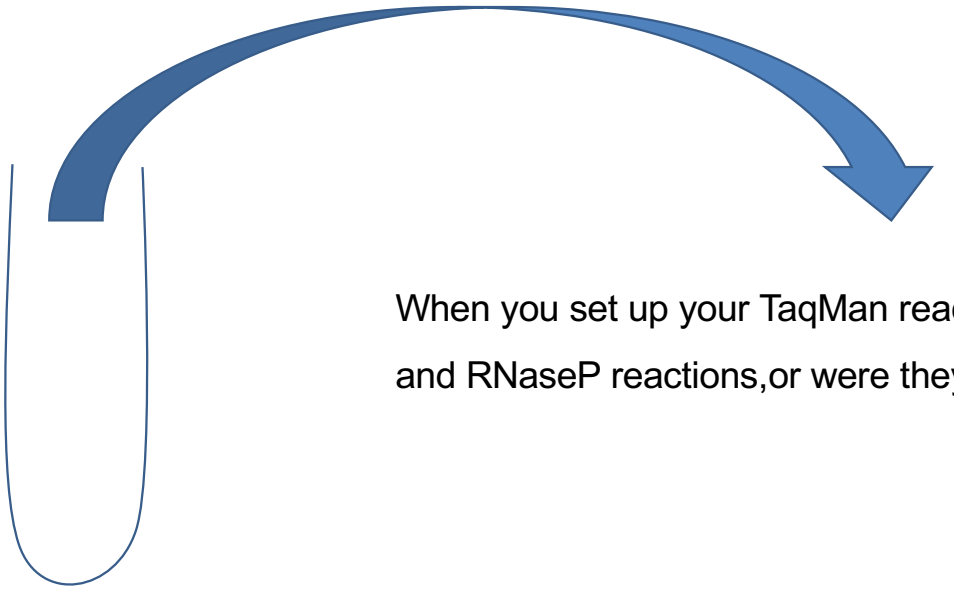
Test for *EGFR* copy number change



RNaseP is our control

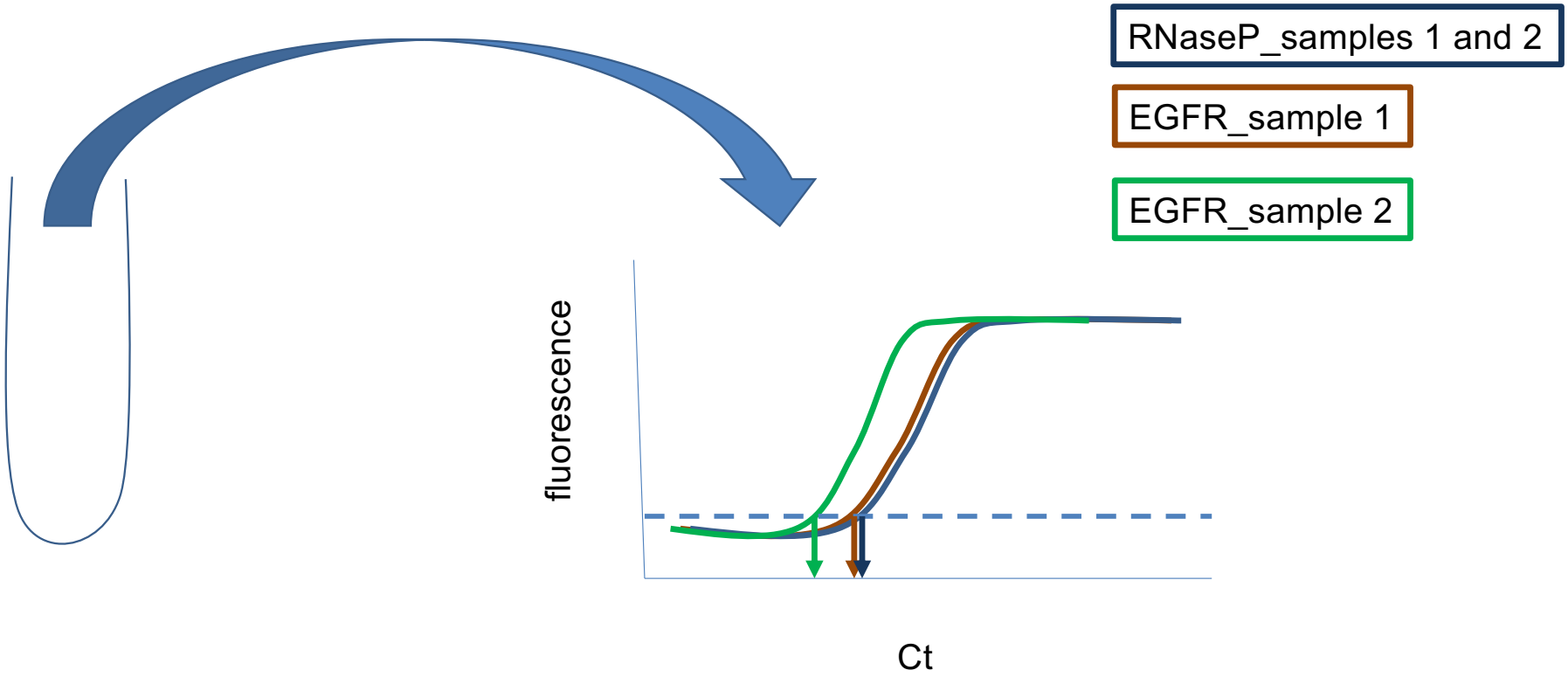
Since *RnaseP* is our control, what is the assumption we are making?

- A key part of the TaqMan methodology is that the EGFR and RNaseP reactions proceed with equal efficiency
- This means we can make a direct comparison between the fluorescence released by each reaction

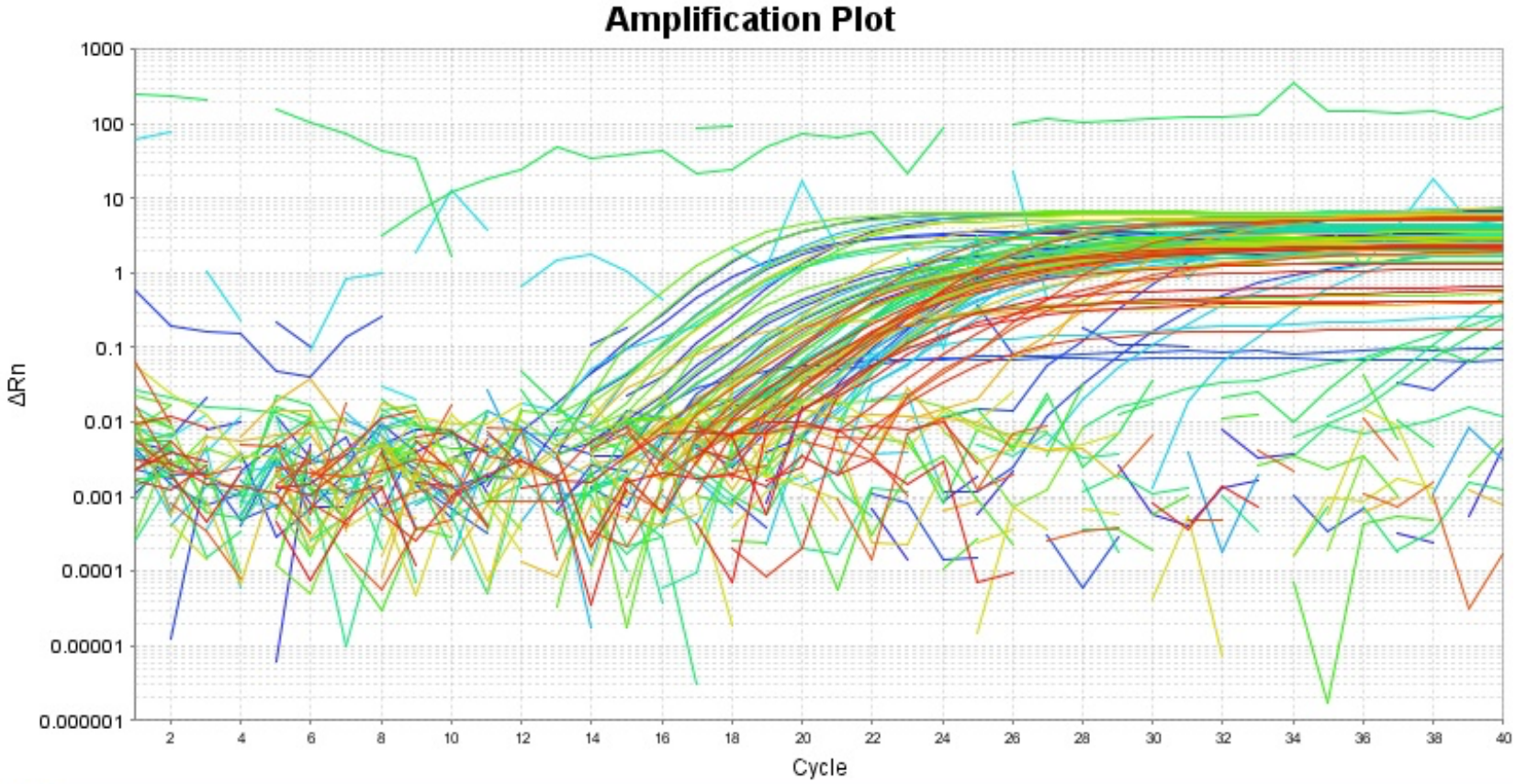


When you set up your TaqMan reaction did it contain the EGFR and RNaseP reactions,or were they done in separate tubes?

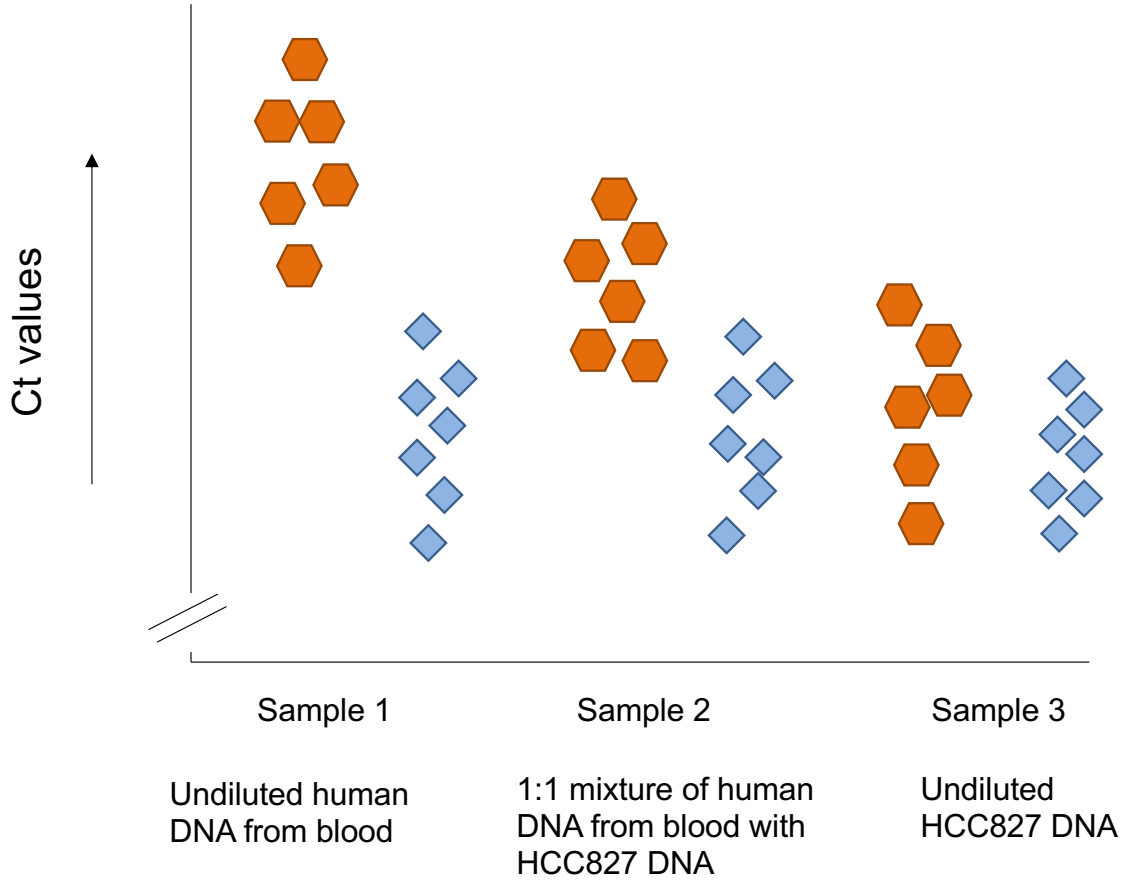
RNaseP is our endogenous control that is amplified at the same efficiency as *EGFR*



How would you interpret this graph?

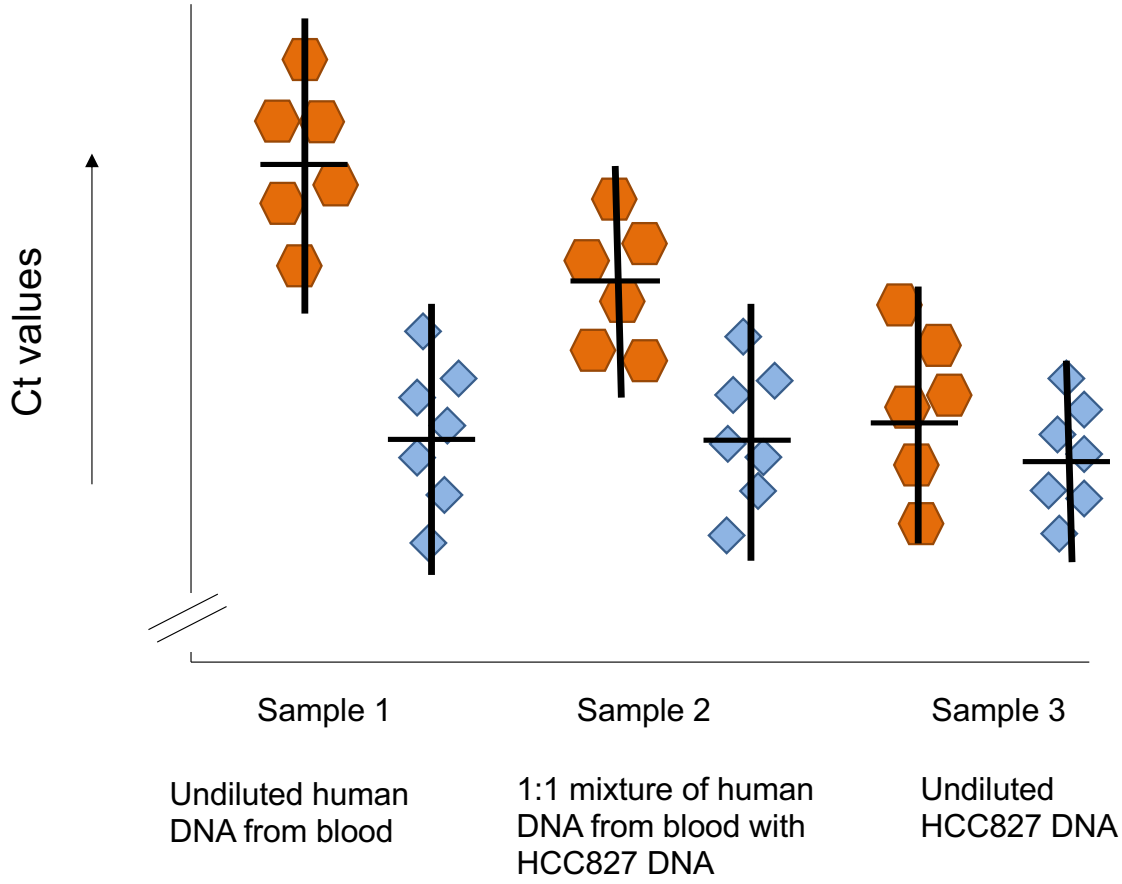

Amplification Plot
ΔRn
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle

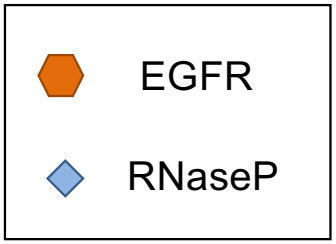
How would you interpret this graph?



Using TaqMan data generated in this class, you will need to plot this graph for your laboratory report

How would you interpret this graph?

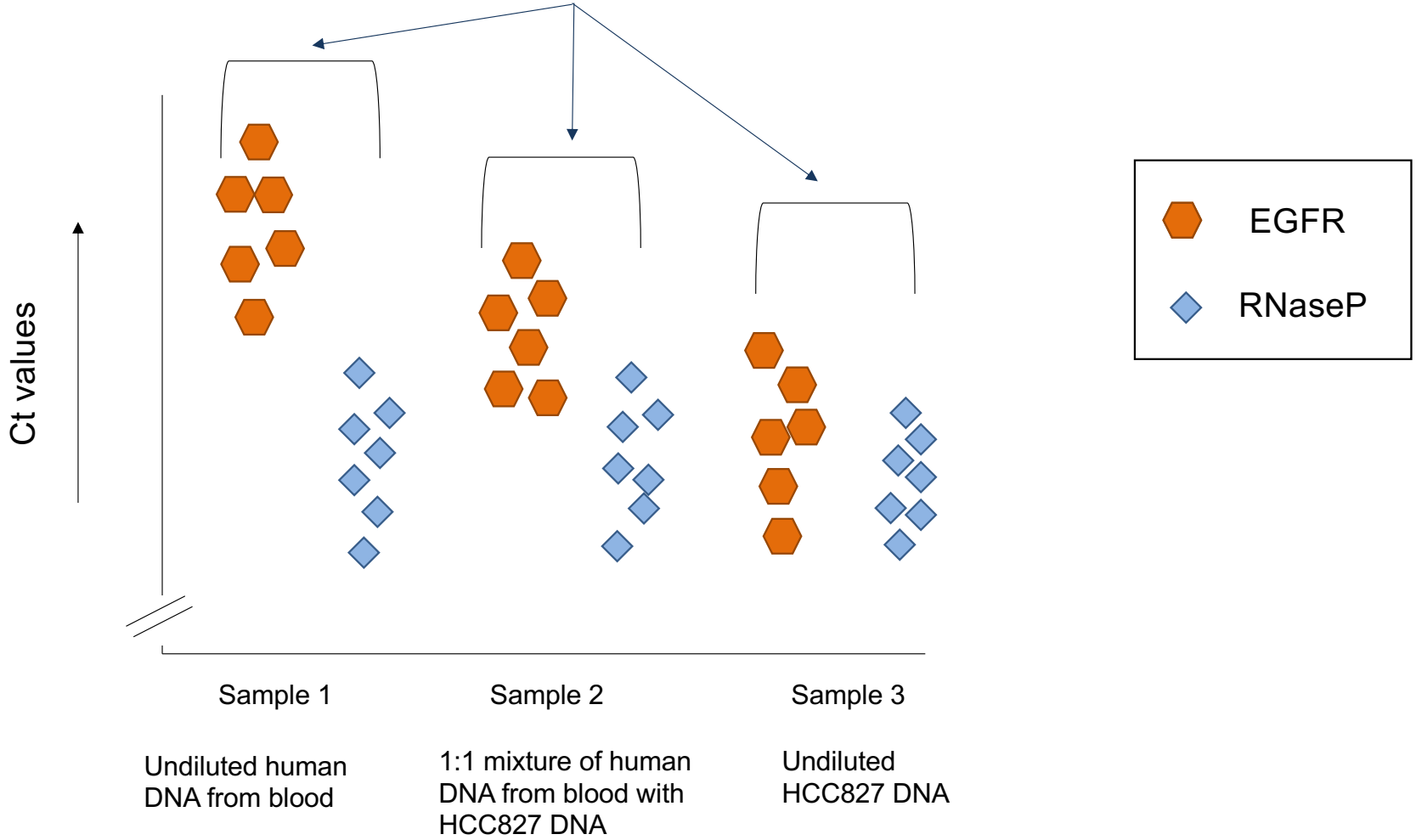




Using TaqMan data generated in this class, you will need to plot this graph for your laboratory report – including lines indicating standard deviation and mean

No column graphs in the report

Is there a significant difference between the RNaseP and EGFR paired samples?



We will use the Mann-Whitney test to test between the values of *RNAseP* and *EGFR* for each sample

You will need to analyse your TaqMan data using the Mann-Whitney test and include it in your laboratory report

- The field of statistics exists because it is usually impossible to collect data from all individuals of interest (population)
- Our only solution is to **collect data from a subset (sample)** of the individuals of interest, but our real desire is to know the "truth" about the population.
- Quantities such as **means, standard deviations and proportions** are all important values and are called **"parameters"** when we are talking about a **population**
- Since we usually cannot get data from the whole population, **we cannot know the values of the parameters for that population**
- We can, however, **calculate estimates of these quantities for our sample**
- When they are calculated from sample data, these quantities are called "statistics." **A statistic estimates a parameter**

Parametric and Nonparametric: Demystifying the Terms
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- **Parametric** statistical procedures rely on assumptions about the shape of the distribution (i.e., assume a normal distribution) in the underlying population and about the form or parameters (i.e., means and standard deviations) of the assumed distribution
- **Nonparametric** statistical procedures rely on no or few assumptions about the shape or parameters of the population distribution from which the sample was drawn.

Parametric and Nonparametric: Demystifying the Terms
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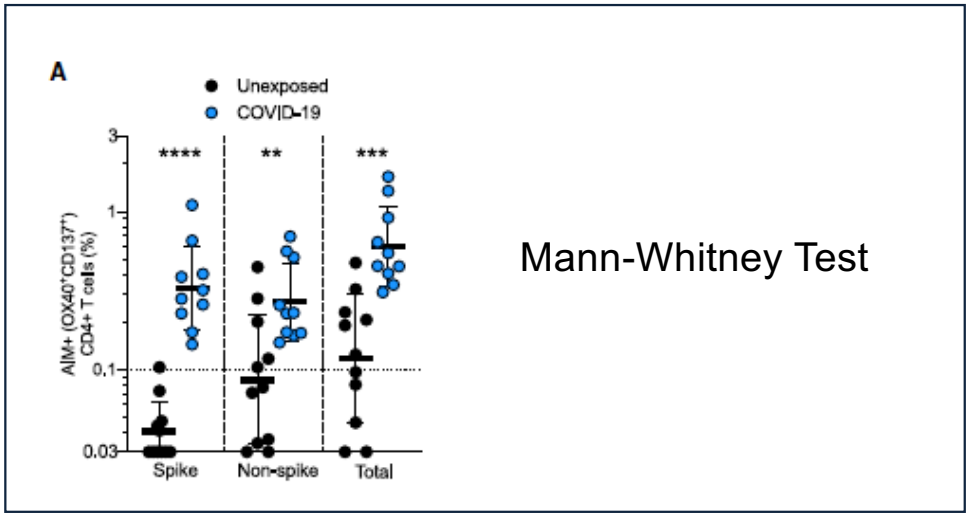
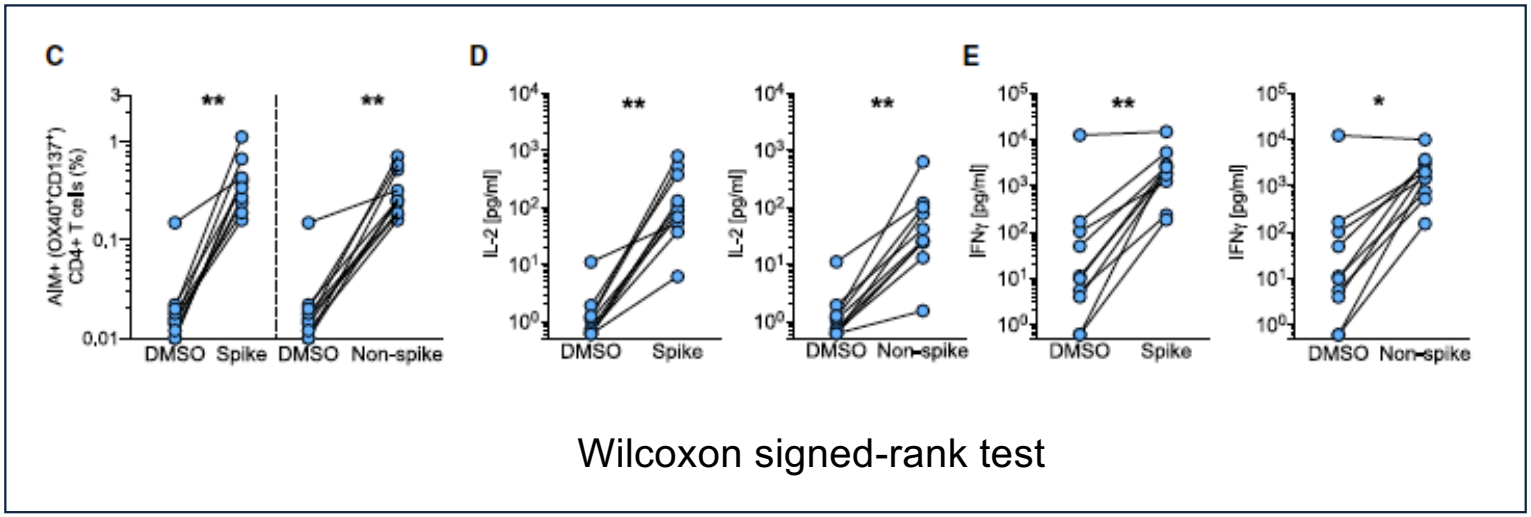
Analysis Type	Example	Parametric Procedure	Nonparametric Procedure
Compare means between two distinct / independent groups	Is the mean percentage of activated T-cells in unexposed versus infected individuals the same?	Two-sample t-test	Mann-Whitney rank-sum test
Compare two quantitative measurements taken from the same individual	Within the same sample, is there a difference between control/untreated versus treated conditions (i.e. DMSO Vs peptide)	Paired t-test	Wilcoxon signed-rank test

- Parametric tests are based on assumptions about the distribution of the underlying population from which the sample was taken
- The most common parametric assumption is that data are approximately normally distributed
- Nonparametric tests do not rely on assumptions about the shape or parameters of the underlying population distribution
- The parametric assumption of normality is particularly worrisome for small sample sizes ($n < 30$). Nonparametric tests are often a good option for these data
- Nonparametric procedures generally have less power for the same sample size than the corresponding parametric procedure if the data truly are normal.



Mann-Whitney Test

What is the Null hypothesis?
What is the alternate hypothesis?



Wilcoxon signed-rank test

Grifoni et al., 2020, Cell *181*, 1489–1501
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Your report

1. Plot the TaqMan data
2. Calculate mean, standard deviation
3. Perform Mann-Whitney test
4. Estimate difference in copy number between Samples 1, 2 and 3